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13. ABSTRACT (Maximum 200 Words)

The proposal ***objective*** is to define the interaction between Notch and VEGFR-3 in breast cancer. Thus, we proposed to investigate this relationship in four different settings: primary endothelial cell cultures, mouse embryos, human breast tumors, and mouse mammary tumor xenografts. In the first year, we have completed the characterization of Notch regulation of VEGFR-3 in primary endothelial cells and begun the evaluation of Notch and VEGFR-3 expression in mouse embryos and human breast cancers. Our preliminary data demonstrated that expression of an activated form of Notch4 increased VEGFR-3 transcripts in three different human primary endothelial cells. Using cell surface biotinylation and immunoblotting, we have found that activated forms of both Notch1 and Notch4 induced VEGFR-3 protein in the endothelial cell lines. This induction of VEGFR-3 *in vitro* was also observed in embryos in which Notch4 was activated within the endothelium. In contrast, the expression of VEGF-C, a ligand for VEGFR-3, was unaffected by Notch signal activation; while, VEGF-D was not expressed, nor induced by Notch in the endothelial cell lines examined. Initial experiments indicate that Notch1, Notch4 and VEGFR-3 are expressed within the vasculature of normal breast and ductal carcinomas, as well as the ductal epithelial cells.

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Notch, VEGF, breast cancer, angiogenesis, lymphangiogenesis

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Introduction

Expression studies have shown that the angiogenic/lymphatic factor VEGFR-3, its ligand VEGF-C and the Notch ligand, Delta4 are up-regulated in the invading blood and lymphatic vessels in human breast cancers [1, 2]. Using mouse models, Notch signaling has been found to be essential for angiogenesis to progress to completion in the developing embryo [3]. However, the exact mechanism(s) by which Notch signaling regulates vasculogenesis and/or lymphangiogenesis is not well understood. Though, it is known that Notch modulates cell-fate decisions by regulating the expression of tissue specific genes. As many angiogenic regulators have been identified, we have focused on defining the interactions between Notch and that of known angiogenic regulators. Using quantitative RT-PCR analysis, we found that both VEGFR-3 and Delta4 were induced in response to an activated form of Notch4 (Notch4/int-3) in three human primary endothelial cells (HUVEC-umbilical vein, HUAEC-umbilical artery, & HMVEC-dermal microvascular). Taken together these data suggested a relationship between Notch signaling and VEGFR-3. Thus, we ***hypothesized*** that Notch may regulate blood and/or lymphatic vessel development via its induction of VEGFR-3 during physiological angiogenesis, as well as pathological angiogenesis and/or lymphangiogenesis in breast cancer. The ***overall objective*** of this proposal is to define the interaction between Notch and VEGFR-3 in breast cancer. In the first year, we proposed to study their relationship in three different settings: primary human endothelial cell cultures, mouse embryos, and human breast tumors.

Body

Notch1 and Notch4 signal activation up-regulates VEGFR-3 at surface of endothelial cells

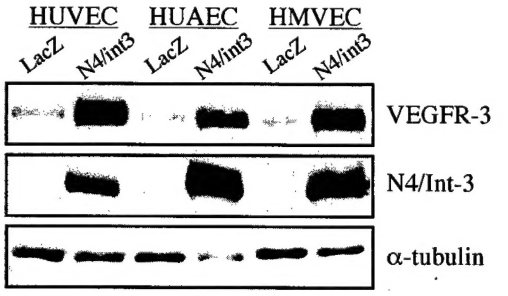

Fig.1 Notch4 signaling up-regulates VEGFR-3 expression on the surface of endothelial cells. HUVEC, HUAEC and HMVEC were infected with Ad-LacZ or Ad-N4/Int-3 and surface biotinylated 24 hours post infection. Western analysis of streptavidin purified surface proteins using an antibody against VEGFR-3. Western analysis of total lysates using 12CA5 (an antibody against the HA tag of N4/int-3) and anti-α-tubulin.

Previously, we had shown that the ectopic expression of a truncated and constitutively active allele of Notch4, N4/Int-3 strongly induced the expression of VEGFR-3 transcripts in three different human primary endothelial cell lines [human umbilical vein endothelial cells (HUVEC), human umbilical artery endothelial cells (HUAEC) and human neonatal dermal microvascular endothelial cells (HMVEC)]. HUVEC and HUAEC are endothelial cells isolated from specialized large vessels. Whereas, HMVEC are derived from dermal capillaries and are comprised of both blood and lymphatic endothelial cells. To further characterize the interaction between Notch4 and VEGFR-3, we determined if the induction of VEGFR-3 mRNA by Notch4 correlated with an up-regulation of VEGFR-3 protein. HUVEC, HUAEC and HMVEC were infected with adenoviruses encoding either N4/Int-3 or LacZ (control) at a moi of 25 pfu/cell and 48 hours later surface-biotinylated. The surface proteins were purified with streptavidin beads and Western analysis performed using an antibody against VEGFR-3 (Fig. 1). Western analysis was performed on total cell lysates to confirm the expression of N4/int-3 and α-tubulin as a control for protein concentration of the lysates. All three endothelial cell lines infected with Ad-LacZ expressed a low level of VEGFR-3. Consistent with an increase in VEGFR-3 transcripts in response to Notch4 signal activation, N4/Int-3 up-regulated the expression of VEGFR-3 at the cell surface of HUVEC, HUAEC and HMVEC.

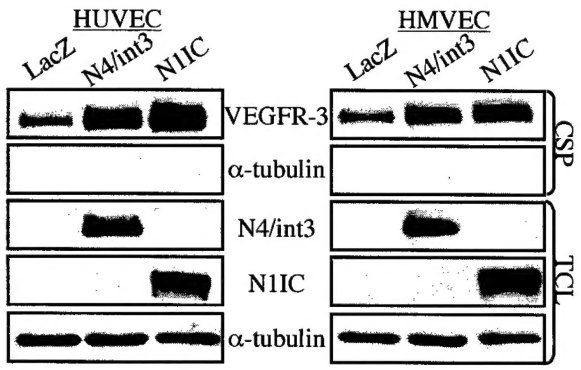

Fig2. Notch4 and Notch1 both induce VEGFR-3 expression on the surface of HUVEC and HMVEC. HUVEC and HMVEC were infected with Ad-LacZ, Ad-N4/Int-3 or Ad-N1IC and surface biotinylated 24 hours post infection. Western analysis of streptavidin purified surface proteins using an antibody against VEGFR-3 and α-tubulin. Western analysis of total lysates using 12CA5 (an antibody against the HA tag of N4/int-3) and antibodies against Notch1 and α-tubulin.

Since loss of function studies in mice suggested that both Notch1 and Notch4 have an overlapping role in embryonic angiogenesis, we wanted to determine if Notch1 signaling could also up-regulate VEGFR-3. To activate Notch1, we used a constitutively active human Notch1 adenoviral construct that encodes the cytoplasmic domain of Notch1 (N1IC). HUVEC and HMVEC were infected with Ad-N1IC, Ad-N4/Int-3 or Ad-LacZ and cell surface biotinylation performed 24 hours post-infection. Streptavidin purified and total cell lysates were analyzed by immunoblotting (Fig 2). Both Notch1 and Notch4 signal activation up-regulated VEGFR-3 to a similar level.

Notch signaling up-regulates VEGFR-3 in blood endothelial cells

VEGFR-3 has been suggested to function in both angiogenesis and lymphangiogenesis. HMVEC isolated from dermis are comprised of both blood (BEC) and lymphatic (LEC) endothelial cells [20]. Since HUVEC and HUAEC are comprised solely of BECs and Notch induced VEGFR-3 in these primary endothelial cell lines, this suggested that Notch was up-regulating VEGFR-3 in the BEC population of HMVEC. We further wanted to determine if Notch induced VEGFR-3 in a subset of cells or if Notch signaling increased the number of cells expressing high levels of VEGFR-3. BECs purified from HMVEC [4] were infected with adenoviruses for N1IC, N4/Int-3 or LacZ. The following day, Ad-infected BECs were analyzed by FACS for VEGFR-2 and VEGFR-3 expression. The percentage of cells expressing VEGFR-2 remained unchanged between LacZ and Notch expressing BECs (Table 1). In contrast, Notch1 and Notch4 signal activation increased the number of VEGFR-3 expressing BECs. FACs analysis was also performed on LECs infected with adenoviruses encoding activated forms of Notch or LacZ. However, the percentage of parental LECs expressing VEGFR-3 is very high and was unaffected by Notch activation (data not shown). We are currently attempting to inhibit endogenous Notch signaling by expressing a Notch antagonist in LECs to determine if VEGFR-3 expression is perturbed.

	LacZ	Notch1IC	Notch4/Int3
VEGFR-2	77.31%	78.34%	79.76%
VEGFR-3	40.08%	76.71%	70.61%

Table 1. Notch signaling up-regulates VEGFR-3 in BECs isolated from HMVEC. BECs were infected with either Ad-LacZ, Ad-N1IC or Ad-N4/int3 and 24 hours post infection FACS was performed using antibodies against VEGFR-2 and VEGFR-3. Data represented as percentage of cells expressing either receptor.

The induction of VEGFR-3 by Notch in blood endothelial cells indicates that Notch may function to regulate VEGFR-3 in angiogenesis and not lymphangiogenesis. To examine this possibility, HMVEC were infected with adenoviruses for N1IC, N4/Int-3 or LacZ and quantitative RT-PCR performed to determine the transcript levels of LEC specific genes, Prox1, podoplanin and LYVE-1. Interestingly, Notch1 signaling down-regulated Prox1 and podoplanin and Notch4 induced LYVE-1 (Fig. 3). These preliminary results suggest a possible separation in function for Notch1 and Notch4 in regulating VEGFR-3 expression. Notch1 may promote angiogenesis by inhibiting the lymphangiogenic

function of VEGFR-3. In contrast, Notch4 may promote a VEGFR-3 function in lymphangiogenesis. Further experiments are necessary to investigate this model and are currently being done. Purified LECs and BECs will be infected with adenoviruses encoding either N1IC, N4/int-3 or LacZ and analyzed by quantitative RT-PCR, FACS and immunoflourescence for alteration in the expression of the LEC specific genes, as well as BEC specific genes.

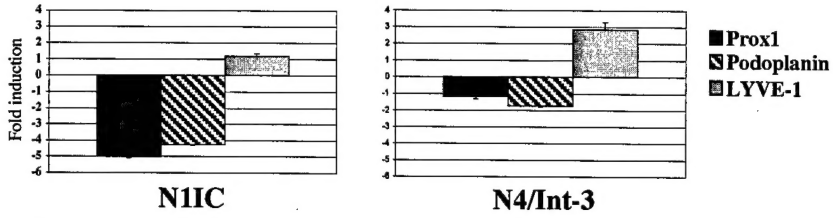

Figure 3. Notch1 and Notch4 differentially regulate lymphatic endothelial specific genes. Quantitative RT-PCR of HMVEC infected with Ad-LacZ Ad-N1IC or Ad-N4/Int-3. Data represented as fold induction of Ad-N1IC or Ad-N4/Int-3 relative to Ad-LacZ Control.

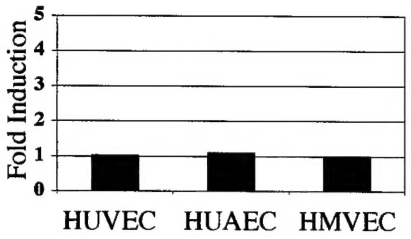

Figure 4. Notch4 signal activation does not alter VEGF-C expression. Quantitative RT-PCR of HUVEC, HUAEC, and HMVEC infected with either Ad-LacZ or Ad-N4/Int-3. Data represented as fold induction of VEGF-C in Ad-N4/Int-3 relative to Ad-LacZ Control.

Notch signaling does not affect the expression of VEGFR-3 ligands, VEGF-C and VEGF-D

We also wanted to ascertain if the Notch specific induction of VEGFR-3 corresponded with an up-regulation of the ligands for VEGFR-3, VEGF-C and VEGF-D. To determine their expression, the three primary endothelial cells lines were infected with Ad-LacZ or Ad-N4/int-3, and quantitative RT-PCR performed. VEGF-C was expressed by all three primary endothelial cell lines, but was not affected by Notch4 activation (Fig. 4). In contrast, VEGF-D was not expressed by any of the primary endothelial cells, nor induced by Notch (data not shown)

Notch signaling within the vascular endothelium induces aberrant VEGFR-3 expression

Having established that Notch4 activity induces VEGFR-3 expression *in vitro*, we next wanted to confirm this observation *in vivo* by immunohistochemical analysis of mouse embryos in which Notch activity has been altered. In collaboration with Janet Rossant at the Samuel Lunenfeld Research Institute, Toronto, we have previously demonstrated that transgenic embryos in which Notch4 has been constitutively activated specifically within the vascular endothelium under control of the VEGFR-1 promoter ($Tie1^{+/LacZ}$, $Flk1^{+/Notch4/int-3}$) die at day 9.5 of embryonic development due to hemorrhaging and vascular disorganization [5]. To determine if Notch4 activation within the endothelium correlated with an induction of VEGFR-3, $Tie1^{+/LacZ}$, $Flk1^{+/Notch4/int-3}$ and phenotypically normal $Tie1^{+/LacZ}$ E9.0 embryos were provided by the Rossant laboratory. Wholemount immunohistochemistry was performed using antibodies against the vascular endothelial cell marker, PECAM and VEGFR-3 (Fig. 5). We chose to analyze less severely affect double transgenic embryos so that differences in VEGFR-3 expression were due to Notch activation and not a difference in the number of endothelial cells. As compared to $Tie1^{+/LacZ}$ controls, $Tie1^{+/LacZ}$, $Flk1^{+/Notch4/int-3}$ embryos have less PECAM staining in the head and rombomeres consistent with a loss of capillary vessels due to defects in angiogenesis. In contrast, the large intersomitic vessels do not appear to be as affected in the $Tie1^{+/LacZ}$, $Flk1^{+/Notch4/int-3}$ embryos. Consistent with our *in vitro* data, VEGFR-3 was strongly expressed in the intersomitic vessels of the $Tie1^{+/LacZ}$, $Flk1^{+/Notch4/int-3}$ embryos as compared to the $Tie1^{+/LacZ}$ control embryos. Since $Tie1^{+/LacZ}$, $Flk1^{+/Notch4/int-3}$ embryos precociously express VEGFR-3 within the embryonic vasculature, we still need to determine whether LEC specific genes are also misexpressed. Wholemount immunostaining will be

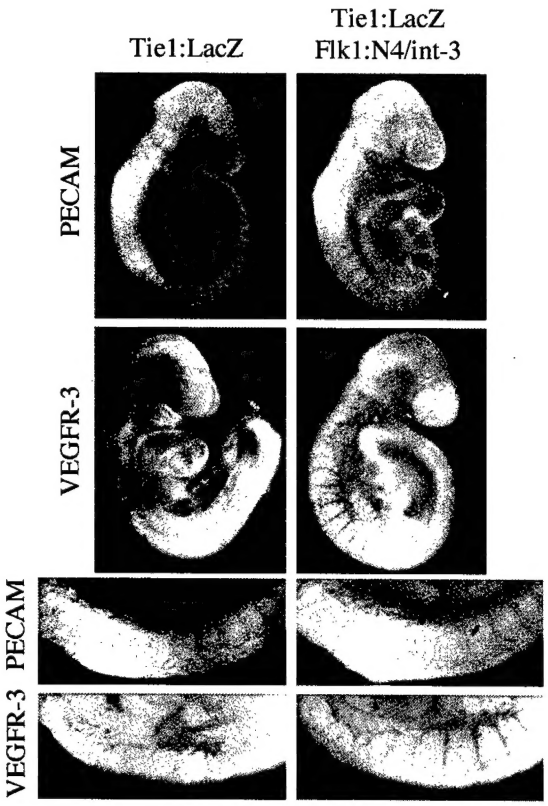

Figure 5. Notch4 signal activation in the embryonic endothelium results in precocious VEGFR-3 expression. Wholemount immunostaining of E9.0 Tie1+/LacZ and Tie1+/LacZ, Flk1+N4/LacZ embryos using antibodies against the vascular endothelial cell marker, PECAM and VEGFR-3. Lower panels are magnified views of intersomitic vessels.

performed with antibodies against Prox1, podoplanin and LYVE-1. Mice nullizygous for both Notch1 and Notch4 also die at E9.5 due do defects in vascular remodeling [6]. We have re-established these lines in our laboratory and currently generating double homozygous null embryos for wholemount immunohistochemical analysis. We expect that endothelial expression of VEGFR-3 will be disrupted in these double nullizygous embryos.

Expression of Notch1, Notch4 and VEGFR-3 in normal breast and ductal carcinoma tissue

We have begun preliminary work to determine if VEGF-R3 and Notch4 are co-expressed in the vasculature of human breast tissues. Immunostaining of 5micron serial sections revealed that VEGFR-3 and Notch4 are co-expressed in the vasculature of wild-type breast tissue (Fig. 6). VEGFR-3 and Notch4 was only expressed in a sub-set of the vessels, as compared to PECAM immunostaining which markers all vascular endothelial cells. Interestingly, Notch4 and VEGFR-3 were also co-expressed in the wild-type ductal epithelial cells. We have also begun to immunostain human ductal breast carcinomas (Fig. 7). Notch1 and Notch4 were expressed in the neovessels surrounding the tumor cells. Botch Notch proteins were also expressed in the transformed ductal cells. Notch1 was expressed in the infiltrating lymphocytes which is consistent with a known role for Notch in lymphocyte differentiation [7]. We have yet to determine if these vessels are blood or lymphatic. Immunohistochemistry using antibodies against CD34 and LYVE-1 will be performed to discriminate between blood and lymphatic endothelial cells, respectively. Immunostaining for Notch1, Notch4 and VEGFR-3 is currently being performed on additional ductal carcinomas and will be extended to other breast tumor types. We are planning to perform additional immunohistochemistry analysis to determine the expression of Delta4,

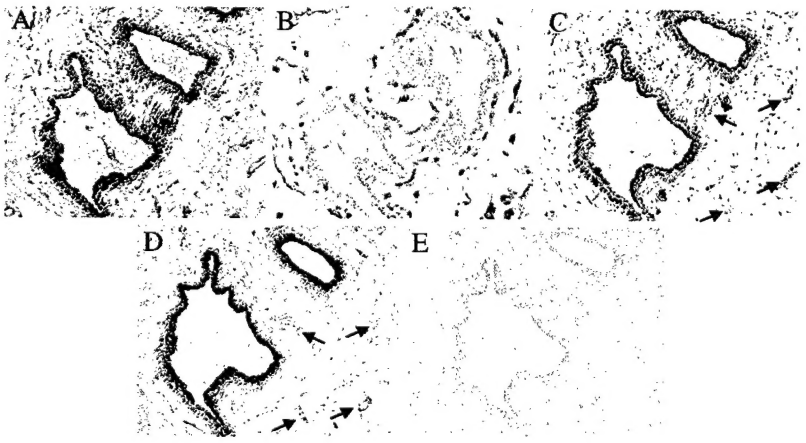

Figure 6. Immunostaining of normal breast tissue.
A. Hemotoxylin and eosin. **B.** PECAM (vascular endothelial cell marker). **C.** Notch4. **D.** VEGFR-3. **E.** VEGFR-3 with VEGFR-3 blocking antibody. Red arrows indicate vessels.

Notch1, VEGF-C and VEGF-D in normal and tumor breast tissues.

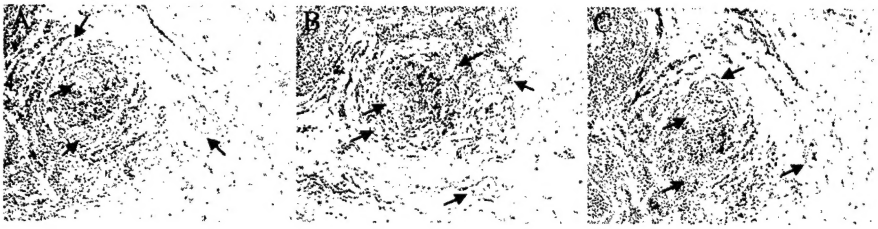

Figure 6. Immunostaining of mammary ductal carcinoma tissue.
A. PECAM (vascular endothelial cell marker). **B.** Notch1. **C.** Notch4. Red arrows indicate vessels. Black arrows indicate tumor cells. Blue arrow indicates lymphocytes.

Key Research Accomplishments

- Confirmed Notch1 and Notch4 up-regulates VEGFR-3 at the cell surface of primary endothelial cells (HUVEC, HUAEC, HMVEC) consistent with our RT-PCR data.
- Using FACS, demonstrated that Notch up-regulates VEGFR-3 in blood endothelial cells (BEC) isolated from HMVEC.
- Using embryos in which Notch signaling is specifically induced within the vascular endothelium, demonstrated that Notch4 induced precocious expression of VEGFR-3 within the vasculature.
- Demonstrated that Notch1, Notch4 and VEGFR-3 are expressed within the vasculature of normal breast tissue and breast ductal carcinoma tissue.

Reportable Outcomes

Publications (submitted prior to award, but published during award period)

Shawber, C.J., and Kitajewski, J. (2004) Notch function in the vasculature: Insights from Zebrafish, mouse and man. *BioEssays*. 26: 225-234.

Shawber, C.J., Das, I., Francisco, E., and Kitajewski, J. (2003) Notch signaling in primary endothelial cells. *Ann. N. Y. Acad. Sci.* 995:162-170.

Abstracts

Shawber, C., Funahashi, Y., Franscisco, E., Podgrabinska, S., Voronchikhina, M., Shiraishi, K., Chawengsaksophak, K., Rossant, J., Skobe, M. and Kitajewski, J. (2004) *Identification and Characterization of Notch Regulated Genes in Blood and Lymphatic Endothelial Cells.* Molecular Mechanisms in Lymphatic Function and Disease. Gordon Research Conference, Ventura, California.

Awards and Honors

2004 Lymphatic Research Foundation-Andrew Moisoff Young Investigator Poster Award Recognition, Molecular Mechanisms in Lymphatic Function and Disease, Gordon Research Conference, Ventura, California.

Conclusions

The proposal ***objective*** is to define the interaction between Notch and VEGFR-3 in breast cancer. In year one, we investigated this relationship in three different settings: primary endothelial cell cultures, mouse embryos, and normal and malignant human breast tissue. We have completed the characterization of Notch regulation of VEGFR-3 in primary endothelial cells and begun the evaluation of Notch and VEGFR-3 expression in mouse embryos and human breast cancers. We confirmed that the observed induction of VEGFR-3 transcripts by Notch4 signal activation correlated with an up-regulation of VEGFR-3 protein at the cell surface of the primary endothelial cell lines, HUVEC, HUAEC and HMVEC. Similar to Notch4 signaling, Notch1 activation induced VEGFR-3 protein in HUVEC and HMVEC. Using FACS analysis of BEC purified from HMVEC, we found that Notch increased the percentage of VEGFR-3 expressing blood endothelial cells. Interestingly, quantitative RT-PCR of HMVEC expressing either N1IC or N4/Int-3 suggests a separation in function for Notch1 and Notch4 in regulating angiogenic and lymphangiogenic function of VEGFR-3. In year two, this result will be examined further. The induction of VEGFR-3 *in vitro* by Notch also occurred in embryos in which Notch4 was activated within the embryonic endothelium. In the primary endothelial cell cultures, the expression of VEGF-C, a ligand for VEGFR-3, was unaffected by Notch signal activation; while, VEGF-D was not expressed, nor induced by Notch. Initial experiments indicate that Notch1, Notch4 and VEGFR-3 are expressed within the vasculature of normal breast and ductal carcinomas, as well as the ductal epithelial cells. In year two, we will extend this analysis to ductal, intraductal, and lobular carcinomas, and fibroadenomas. Immunohistochemistry using antibodies against VEGF-C, VEGF-D, Delta4 will be performed. To discriminate between blood and lymphatic vessels, immunostaining will also be done with antibodies against CD34 and LYVE-1, respectively.

Complication from metastatic disease is the leading cause of breast cancer-related deaths. In breast cancer, the spread of tumor cells throughout the body is in part dependent on their access to blood and lymphatic vessels. Thus, there is a crucial need to identify the genes involved in regulating angiogenesis and lymphangiogenesis. Previous studies suggest that the angiogenic/lymphatic factor VEGFR-3, its ligand VEGF-C and the Notch ligand Dl4 may regulate these processes in human breast cancer [1, 2]. By examining the relationship between Notch and VEGFR-3 in both physiological and pathological vascular development, we will increase our understanding of these processes and may define new targets for potential cancer therapeutics.

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Notch Signaling in Primary Endothelial Cells

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ABSTRACT: The Notch family of cell-surface receptors has been proposed to regulate cell-fate decisions by modulating the ability of each cell to respond to environmental cues. In vertebrates, gain-of-function and loss-of-function studies have demonstrated a requirement for Notch signaling for proper patterning of the vasculature during embryogenesis. To examine the molecular mechanisms by which Notch regulates vascular development, we analyzed changes in gene expression in response to Notch signaling. Notch signal transduction and function were assessed in primary human endothelial cells isolated from the dermal microvasculature of neonates, HMVECd. We demonstrate that HMVECd cells express a heterodimeric form of endogenous Notch4 on their cell surface. Using an *in vitro* coculture assay, we found that Delta4 can function as a ligand for Notch4 in HMVECd cells. Moreover, ectopic expression of an activated allele of Notch4 upregulated the expression of Delta4, suggesting that there may be a regulatory loop between Notch4 and its ligand, Delta4. Notch4 activation also induced the expression of the transcriptional repressors, HES1, HERP1, and HERP2, as well as ephrinB2, an angiogenic factor proposed to be involved in arterial/venous endothelial cell specification.

KEYWORDS: Notch; Delta; primary endothelial cells; angiogenesis

INTRODUCTION

Notch functions in an evolutionarily conserved signaling pathway that is required for numerous cell-type specifications. In mammals, the Notch family consists of four receptors (Notch 1–4) and has five ligands (Jagged 1–2 and Delta 1, 3, 4).[1] Depending on the environmental context, Notch signaling has been proposed to influence many different types of cell-fate decisions by providing inhibitory, inductive, or proliferative signals (reviewed in refs. 2–4). This pleiotropic function prompts the idea that Notch interfaces with multiple signaling pathways in a spatiotemporal manner in the embryo and adult.

Notch regulates cell-fate decisions by altering patterns of gene expression. Upon ligand activation, the cytoplasmic domain of Notch is proteolytically released and translocates to the nucleus where it interacts with CSL [CBF1, Su (H), Lag-2] transcriptional repressors and converts them to transcriptional activators. Both *in vivo*

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and *in vitro* studies indicate that the HES and HERP families of transcriptional repressors are the direct targets of Notch/CSL-dependent signaling.[5–8] Expression of HES1 or the *Drosophila* homologue E(Spl) has been shown to repress the expression of proneural bHLH transcription factors, preventing neuronal differentiation.[1,7] However, in most other cell types, the genes that HES and HERP gene products regulate have not been identified.

A number of observations suggest that the Notch signaling pathway may play a role in cell-fate determination and patterning of the vascular system. Notch1, Notch4, Jagged1, and Dll4 are all expressed in the developing vasculature, while Notch3 is expressed in the accessory smooth muscle cells.[9–12] Moreover, gain-of-function and loss-of-function studies in vertebrates have demonstrated that Notch is required for proper patterning and development of blood vessel networks during embryogenesis.[11,13,14] Mice nullizygous for Jagged1 or Notch1 and Notch4 die *in utero* due to severe hemorrhaging and vascular disorganization in the embryo, yolk sac, and intersomitic vessels.[11,13] Similarly, exogenous expression of an activated Notch4 in the developing endothelium also results in embryonic lethality due to vascular patterning defects.[14] These data suggest that appropriate levels of Notch signaling are crucial for the development of the embryonic vasculature. Recent experiments have also implicated Notch signaling in arterial/venous specification. Expression analysis of E13.5 mouse embryos found that Notch1, Notch3, Notch4, Delta4, Jagged1, and Jagged2 expression became restricted to the arteries and was absent from the veins.[12] Consistent with the expression data, disruption of Notch signaling in Zebrafish was associated with a loss of the arterial marker ephrinB2, while ectopic expression of an activated form of Notch led to a loss in the venous cell marker EphB4 within the dorsal aorta.[15] Taken together, these data suggest that Notch signaling may function at a number of critical steps during vascular development, including vasculogenesis, angiogenesis, and arterial/venous specification. However, the molecular mechanism(s) by which the Notch signaling pathways influence these different steps of vascular development has yet to be elucidated.

Here, we report our findings using the human primary endothelial cell line derived from neonatal dermal microvasculature (HMVECd). HMVECd cells endogenously express Notch4 and Delta4. In coculture experiments using ligand-expressing fibroblasts and Notch-expressing HMVECd cells, we demonstrate that Delta4 binds and activates Notch4. Finally, we have identified a number of genes whose expression is upregulated in response to Notch4 activation. These genes include Delta4, HES1, HERP1, HERP2, and ephrinB2.

MATERIALS AND METHODS

Constructs

The full-length Notch4 cDNA and the naturally occurring Notch4/int3 cDNA[14] were engineered in the adenovirus expression vector pAd-Lox. Adenoviral stocks were generated and titered as previously described.[16] The full-length Delta4 cDNA fused in frame with a myc/His tag was engineered in the mammalian expression vector pcDNA3.1 (Chiron). For detection with the anti-HA monoclonal 12CA5 antibody, Notch4 constructs have been tagged at their C-terminus with one copy of

the HA epitope. The CBF1 luciferase reporter has been previously described.[17] The LacZ cDNA was engineered into both pAd-Lox and the expression vector pHyTc[18] (Genbank accession numbers for mouse Notch4: U43691; mouse Notch4/int3: M80456; and human Delta4: AF253468).

Cell Lines and Growth Conditions

Human primary endothelial cells derived from the dermal microvasculature of neonates (HMVECd) were purchased from Clonetics (Maryland) and maintained according to manufacturer's protocol. Bosc23[19] cells were maintained in Dulbecco's modified medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin.

Adenoviral Infections and Westerns

Adenoviruses encoding Notch4, Notch4/int3, or LacZ were used to infect HMVECd cells at an m.o.i. of 100 pfu/cell. At an m.o.i. of 100, Ad-LacZ infection led to greater than 95% of the cells being LacZ-positive at 24 hours postinfection. Twenty-four hours postinfection, LacZ and Notch4/int3HA-expressing HMVECd were lysed in RIPA buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% NP40, 1% DOC, 0.1% SDS). Lysates were separated on an 8% SDS/PAGE gel and transferred to nitrocellulose (MSI). Western analysis was performed using the monoclonal anti-HA antibody 12CA5 (Babco) at a 1:1000 dilution in BLOTTO, and proteins were visualized using Enhanced Chemiluminescence (ECL; Amersham) in conjunction with a secondary antimouse IgG-HRP conjugate.

Cell Surface Biotinylation and Westerns

Twenty-four hours postinfection, parental and Notch4-expressing HMVECd cells were incubated with 0.5 mg/mL EZ-Link Sulfo-NHS-Biotin reagent (Pierce) in PBS supplemented with 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1% glucose (PBS-CMG) for 20 min at 4°C. Cells were then incubated with PBS–100 mM glycine for 15 min at 4°C and rinsed 2× with PBS-CMG to wash off excess biotin. Cells were lysed in 300 mM NaCl, 50 mM Tris, pH 8.0, 0.5% NP40, 0.5% DOC, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Lysates were incubated 20 min on ice and cleared. Supernatants were then incubated overnight with 30 μL streptavidin-conjugated beads (Pierce). Streptavidin beads were washed 3× with TENT buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100). Proteins were eluted in boiling sample buffer, separated on a 7.5% SDS/PAGE gel, and transferred to PVDF membrane (Millipore). Membranes were probed with rabbit polyclonal antisera generated against the intracellular domain of Notch 4 (CoRb2-2) as previously described.[18]

Coculture Notch/CSL Reporter Assay

HMVECd cells were plated at 8×10^4 cells/well in 12-well plates. The following day, HMVECd cells were infected with adenoviruses encoding either Notch4 or GFP at an m.o.i. of 100 pfu/cell. Following the adenoviral infection, HMVECd cells were transfected using Lipofectamine 2000 (Invitrogen) containing 1.5 μg CBF1 luciferase reporter and 0.25 μg pHyTc-lacZ. The same day, Bosc23 cells were $CaPO_4$ trans-

fected with 25 μg pcDNA3.1 Delta4 or dsRed (Clonetech) plasmids. Twenty-four hours posttransfection, 4×10^5 parental or Delta4-expressing Bosc23 cells were added to each well of Notch4 or GFP-expressing HMVECd cells. The following day, coculture lysates were collected and assayed for luciferase (Enhanced Luciferase Assay Kit, BD) and β-galactosidase (Galacto-light Plus, Tropix) activity following manufacturers' protocols.

Semiquantitative RT-PCR (sqRT-PCR)

Forty-eight hours postinfection, total RNAs were isolated by guanidium thiocyanate-phenol-chloroform extraction[20] from Notch4/int3 and LacZ-expressing HMVECd cells. One to two μg of total RNA was DNaseI (Ambion) digested followed by first-strand syntheses using random hexamers and Superscript II reverse transcriptase (Invitrogen). PCR primers were designed to recognize both human and murine transcripts for Jagged1, Delta4, HES1, HES5, HERP1, HERP2, ephrinB2, and EphB4. For each gene, human PCR amplicons were subcloned into the pDrive PCR cloning vector (Stratagene) for use as PCR reference standards. PCR reactions were prepared in quadruplicate for each unknown sample, as well as for a matching serially diluted reference standard. Reactions are removed at five cycle intervals, separated on 7% nondenaturing bis-acrylamide gels, and stained with Syber Green (Molecular Probes). Band intensities were determined using Kodak Digital Science: 1D Image Analysis Software. A standard curve for each gene was generated from the reference standard dilution series and the values for the unknown samples were extrapolated. To correct for sample-to-sample variations in RT-PCR efficiency and errors in sample quantitation, sqRT-PCR analysis of β-actin expression was used to normalize the different RNA samples.

RESULTS AND DISCUSSION

Expression of Full-Length and Truncated Forms of Notch4 in HMVECd Cells

We chose to introduce the full-length and truncated Notch4 constructs into human primary endothelial cells derived from the dermal microvasculature of neonates (HMVECd) by adenoviral infection. An advantage of adenoviral vectors is in the ability to infect primary endothelial cell cultures with 100% efficiency. Parental HMVECd cells or HMVECd cells infected with LacZ or full-length Notch4 adenoviruses were biotinylated to analyze the cell-surface expression of Notch4. Biotinylated cell-surface proteins were purified from protein lysates with streptavidin beads, and Westerns were performed. Both full-length and a heterodimeric, furin-processed form of Notch1 have been shown to be present at the cell surface.[21] In the HMVECd cells that ectopically expressed full-length Notch4, both the unprocessed (250-kDa) and processed (70-kDa) forms of Notch4 were detected with antibodies against the cytoplasmic domain of Notch4 (FIG. 1A, lane 2) as compared to the LacZ controls (FIG. 1A, lane 1). Cell surface expression of endogenous Notch4 could also be seen as an approximately 70-kDa band after longer exposure of the Western to film (FIG. 1A, lane 3). A slower migrating band running above the 85-kDa marker was also observed and may represent a phosphorylated or otherwise modified form

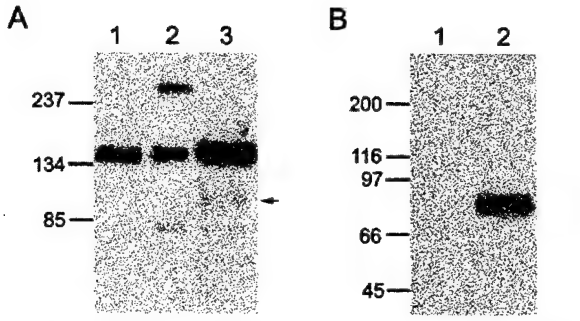

FIGURE 1. Western analysis of endogenous and ectopic Notch4. **(A)** HMVECd cells infected with adenoviruses encoding LacZ (lane 1), full-length Notch4 (lane 2), and parental HMVECd cells (lane 3) were cell-surface biotinylated. Biotinylated Notch4 proteins captured with streptavidin beads were detected by Western analysis using the cytoplasmic polyclonal Notch4 antibody (CoRb2-2). In lane 2, an unprocessed 250-kDa and a processed 70-kDa protein were detected on the surface of HMVECd ectopically expressing Notch4. A longer exposure was necessary to visualize the endogenous Notch4 (lane 3). Both the processed 70-kDa and a slower migrating 85-kDa band (*arrow*) were detected in the parental cells. A strong background band migrates above the 134-kDa marker that is present in both Notch4-expressing and nonexpressing parental cell lines (data not shown). **(B)** Total cell lysates from HMVECd cells infected with adenoviruses encoding either LacZ (lane 1) or Notch4/int3HA (lane 2) were analyzed by Western analysis using the anti-HA monoclonal 12CA5 antibody. Notch4/int3HA migrates as a 70-kDa protein.

of the processed Notch4 cytoplasmic domain.[22] To constitutively activate Notch4 signaling, HMVECd cells were infected with an adenovirus that encodes Notch4/int3HA, a naturally occurring truncated and active allele of Notch4 fused in frame with one copy of the HA epitope tag.[18,23] The expression of the Notch4/int3HA construct was confirmed by sqRT-PCR analysis (data not shown) and Western analysis using the monoclonal 12CA5 antibody that recognizes the C-terminal HA epitope (FIG. 1B, lane 2).

Delta4 Activates Notch4 in HMVECd Cells

The coexpression of Delta4 and Notch4 in the developing vascular endothelium suggests that Delta4 may be a ligand for Notch4. To address this hypothesis, we used an *in vitro* coculture assay to determine if Delta4 could activate Notch4 in HMVECd cells. In this assay, dsRed or Delta4-expressing 293 cells (Bosc23) were cocultured with HMVECd cells infected with adenoviruses encoding either full-length Notch4 or GFP, and then transiently transfected with a CBF1-responsive luciferase reporter and a LacZ expression construct. CBF1 is the mammalian member of the CSL family of transcription factors. Twenty-four hours after coculturing, lysates were collected and assayed for β-gal and luciferase activity. Notch4-expressing HMVECd cells cocultured with Delta4-expressing Bosc23 cells transactivated CBF1 3.8-fold relative to the cocultures with dsRed-expressing Bosc23 cells (FIG. 2). Interestingly, Notch4-expressing HMVECd cells alone or cocultured with the control dsRed-expressing Bosc23 cells also activated CBF1 as compared to the GFP-expressing HMVECd

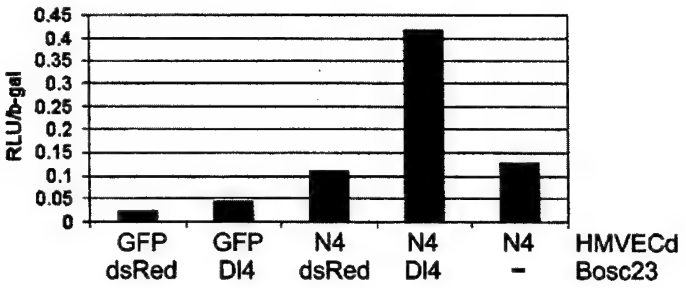

FIGURE 2. Delta4 activates Notch4 in HMVECd cells. To examine whether Delta4 (Dl4) could induce signaling through Notch4 (N4), an *in vitro* coculture assay was used. Dl4 or dsRed-expressing control Bosc23 cells were cocultured with either Notch4 or GFP-infected HMVECd cells that were also transfected with a CBF1 reporter and LacZ plasmid. The data points represent the relative luciferase units (RLU) normalized for transfection efficiency by dividing by the relative β-gal units. Data in this figure represent results from a single coculture experiment. These coculture results have been confirmed in three separate experiments.

cells (FIG. 2). Notch signaling in the absence of ectopic Delta4 expression suggests that Notch4 may have been activated by endogenous ligands. In fact, parental HMVECd cells express both Jagged1 and Delta4 (FIG. 3A). Consistent with our findings in endothelial cells, ectopic expression of both Delta4 and Notch4 in *Xenopus* embryos upregulated the expression of the HES family members ESR-1 and ESR-7 within the neural ectoderm.[9] This proneural phenotype was similar to that seen for activated forms of Notch1. Together with our results, these observations suggest that Delta4 is a ligand for Notch4 in the endothelium.

Identification of Genes Regulated in Response to Notch4 Activation

Studies in vertebrates indicate that Notch signaling is essential for proper vascular development in the embryo. However, the mechanism(s) by which Notch regulates this process has yet to be elucidated. During vascular development, Notch likely modulates cell-fate decisions by regulating the expression of genes involved in Notch signaling and vascular development. Using sqRT-PCR, we examined whether Notch4 signal activation altered the expression of three different categories of genes: Notch ligands (Jagged1 and Delta4), HES/HERP transcriptional repressors (HES1, HES5, HERP1/2), and ephrins (ephrinB2, EphB4).

To activate the Notch pathway in cultured endothelial cells, we have made an adenoviral vector that encodes the int3 allele of Notch4, Notch4/int3. The Notch4/int3 allele is a truncated cytoplasmic form of Notch4 that behaves as a gain-of-function of Notch4 and activates the CBF1-luciferase reporter in cultured endothelial cells (data not shown).[18] We chose to use the constitutively active Notch4/int3 construct for two reasons: (1) the magnitude of Notch4 signaling is greater than that for ligand-activated Notch signaling and (2) coculture assays include ligand-presenting 293 cells that will contribute to the total RNA and obfuscate the data. HMVECd cells were infected with adenoviruses encoding Notch4/int3HA and total RNA was isolated 48

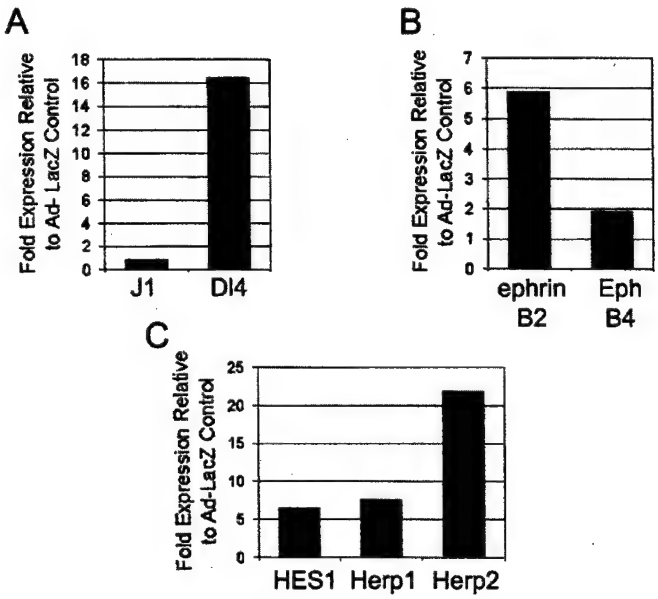

FIGURE 3. Expression analysis of Notch4/int3HA-expressing HMVECd. Semiquantitative (sq) RT-PCR analysis was performed using gene-specific PCR primer pairs for **(A)** the Notch ligands Jagged1 and Delta4, **(B)** the angiogenic factors ephrinB2 and EphB4, and **(C)** the bHLH transcriptional repressors HES1, HERP1, and HERP2. The bar graphs represent the fold-expression of each gene transcript in HMVECd cells expressing Notch4/int3 relative to the HMVECd LacZ control. For each PCR, a standard curve was generated from a serially diluted control plasmid. An R^2 value of greater than 0.93 was achieved for each sqRT-PCR standard curve. Samples were normalized with sqRT-PCR values for β-actin. Data represent an average of two separate sqRT-PCR experiments for each gene examined.

hours postinfection. Adenoviruses encoding LacZ were also used to infect HMVECd cells to control for alterations in gene expression due to the adenoviral infection.

The expression of both Notch4 and its ligand, Delta4, has been shown to be restricted to the developing vasculature in the embryo.[9–12] Consistent with their overlapping expression patterns, Notch4/int3HA expression resulted in a 16-fold increase in endogenous Delta4 expression as compared to the LacZ control (FIG. 3A). In contrast, activation of Notch4 did not significantly alter the expression of Jagged1 (FIG. 3A). Since Delta4 can activate Notch4 in cultured endothelial cells (FIG. 2), this result may suggest a regulatory loop between Notch4 and Delta4 in the vascular endothelium.

Both genetic and biochemical studies have demonstrated that the Notch/CSL complex directly upregulates the expression of the transcriptional repressors of the Hairy Enhancer of Split (HES) and HES-Related Protein (HERP) families in several cell types.[5–8,24] In HMVECd cells, Notch4/int3HA induced the expression of HES1, HERP1, and HERP2 to differing degrees, from 5-fold for HES1 to over 21-fold for HERP2 (FIG. 3C). We also examined if Notch4 signaling altered HES5 expression,

but it was not expressed in HMVECd cells (data not shown). During neuronal differentiation, HES1 has been shown to inhibit the expression of proneuronal bHLH transcription factors.[1,7] Overexpression of HERP2 in human adipose tissue–derived capillary endothelial cells blocked proliferation, migration, and network formation in culture.[25] Thus, Notch4 may also regulate vascular development through a similar mechanism. However, proangiogenic bHLH transcription factors have yet to be identified.

Genetic studies in Zebrafish indicate that Notch signaling is required for the specification of the first embryonic artery by upregulating the expression of the cell-surface ligand ephrinB2 and downregulating the expression of its receptor, EphB4.[15] Consistent with these observations, expression of Notch4/int3HA in HMVECd cells resulted in an almost 6-fold induction in ephrinB2 expression relative to the LacZ controls (FIG. 3B). In contrast, EphB4 expression was relatively unaffected (FIG. 3B). EphrinB2 has been proposed to be critical for the remodeling of the primary vascular plexus and arterial endothelial cell specification.[26] In Zebrafish, the expression of ephrinB2 within arterial endothelium was shown to be dependent on the expression of gridlock, a homologue of HERP1.[27,28] In response to Notch4 activation, we found that HERP1 and HERP2 were induced over 7- and 22-fold, respectively (FIG. 3C). These results suggest that, in HMVECd cells, Notch4 signaling may regulate the expression of ephrinB2 and arterial specification through the induction of HERP1 and/or HERP2.

SUMMARY

Using an *in vitro* coculture assay, we demonstrated that Delta4 can function as a ligand for Notch4 in HMVECd cells. Moreover, expression of Notch4/int3 induced the expression of Delta4, suggesting a regulatory loop between Notch4 and its ligand, Delta4. We also found that Notch4 activation led to an increase in the expression of the transcriptional repressors HES1, HERP1, and HERP2, as well as ephrinB2, an angiogenic factor proposed to be involved in arterial/venous endothelial cell specification. With these analyses, we are beginning to identify the Notch ligand/receptor pairs that function in endothelial cells and some of the target genes regulated by Notch in the vasculature.

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Notch function in the vasculature: insights from zebrafish, mouse and man

Carrie J. Shawber and Jan Kitajewski*

Summary

Vascular development entails multiple cell-fate decisions to specify a diverse array of vascular structures. Notch proteins are signaling receptors that regulate cell-fate determination in a variety of cell types. The finding that Notch genes are robustly expressed in the vasculature suggests roles for Notch in guiding endothelial and associated mural cells through the myriad of cell-fate decisions needed to form the vasculature. In fact, mice with defects in genes encoding Notch, Notch ligands, and components of the Notch signaling cascade invariably display vascular defects. Human Notch genes are linked to Alagille's Syndrome, a developmental disorder with vascular defects, and CADASIL, a cerebral arteriopathy. Studies in zebrafish, mice and humans indicate that Notch works in conjunction with other angiogenic pathways to pattern and stabilize the vasculature. Here, we will focus on established functions for Notch in vascular remodeling and arterial/venous specification and more speculative roles in vascular homeostasis and organ-specific angiogenesis. *BioEssays* 26:225–234, 2004.

The Notch family and its ligands

Notch and its ligands participate in an evolutionary conserved signaling pathway that functions to modulate cell-fate decisions of a variety of cell types originating from all three germ layers.[1–3] *Drosophila* genetics initially identified Notch as a neurogenic gene; that is, loss of Notch was associated with a gain in neurons.[4,5] Analysis of a *C. elegans* Notch, lin-12, helped establish that Notch activity controls binary cell-fate decisions during development.[6,7] In *Drosophila*, a single Notch gene product can be activated by two distinct ligands, Serrate and Delta. In mammals, these families have expanded to four Notch genes (Notch1–Notch4) and five ligands, two Serrate-like (Jagged1, Jagged2) and three Delta-like (Delta-like1, Delta-like3 and Delta-like4).[8–17] Studies in vertebrates have implicated a role for Notch signaling in neurogenesis,[18,19] retinal development,[20–22] somitogenesis;[23–25] adipogenesis,[26] limb development,[27] mygogenesis,[18,28,29] and hematopoiesis.[30–36] In addition, mutations in Jagged1, Notch1, Notch1/Notch4 or components of the Notch signaling cascade in mice result in embryonic lethality associated with severe vascular defects,[37–39] and zebrafish genetics have demonstrated that Notch functions in arterial/venous specification.[40,41]

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Abbreviations: bHLH, basic helix-loop-helix; SEL-10, suppressor and/or enhancer of lin-12; VEGF, vascular endothelial growth factor; Ang, angiopoietin; PDGF, platelet-derived growth factor; E, embryonic day; Flt4, fms-related tyrosine kinase 4.

Notch signal transduction

Depending on the cellular context, Notch signaling has been found to inhibit as well as induce differentiation, induce proliferation and promote cell survival.[3,42,43] The Notch signaling pathway modulates this diverse array of cell-fate decisions by regulating the expression of genes in a cell-type-specific manner. Both receptors and ligands are membrane-spanning, cell surface proteins (Fig. 1). The cell surface localization of both ligand and receptor is consistent with their regulation of cell-fate decisions via direct cell–cell interactions. Notch proteins exist as heterodimeric receptors with an extracellular and an intracellular peptide held together by non-covalent interactions (Fig. 1). This processing of Notch occurs via a furin-like protease prior to ligand activation.[44] Upon ligand-binding, the cytoplasmic domain of Notch is released from the cell surface by a presenilin-dependent proteolytic cleavage.[44,45] The intracellular Notch peptide then translocates to the nucleus, interacts with the CSL (CBF, Su (H), Lag-2) transcriptional repressor, and converts it to a transcriptional activator.[44,46–48] The Hairy/Enhancer of Split (HES) and HES-related (Hey, CHF, HRT, HESR) genes are the direct targets of Notch/CSL-dependent signaling.[49,50] The HES and Hey genes constitute two subfamilies of the bHLH-Orange domain family of transcriptional repressors. Notch also signals by a CSL-independent pathway, and recent studies in *Drosophila* suggest that this pathway is dependent on the transcription factor Deltex.[28,29,51] Negative regulation of Notch is accomplished in part by a SEL-10/

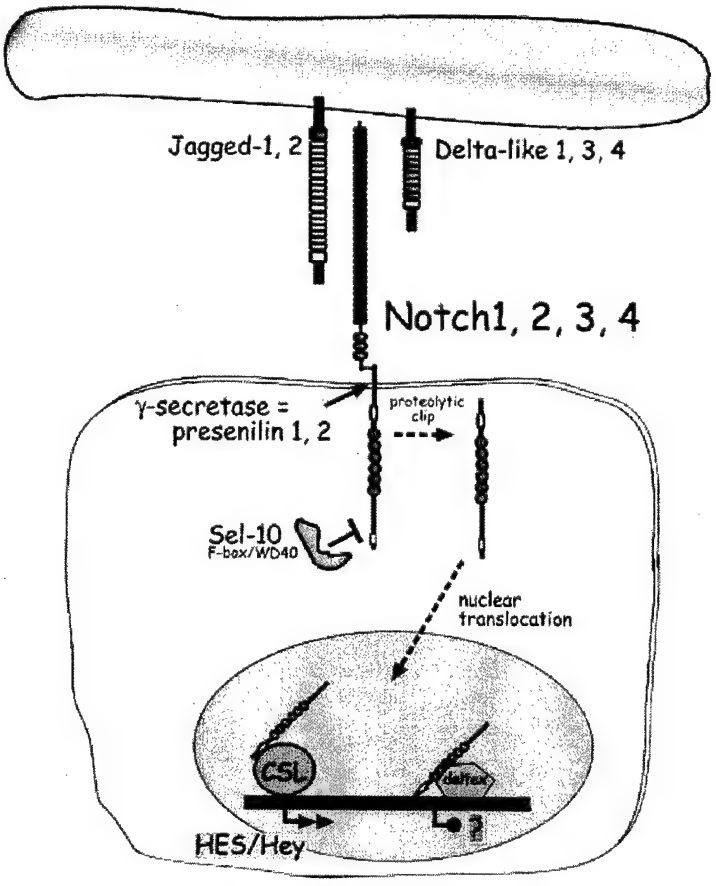

Figure 1. Schematic of Notch signal transduction.

ubiquitin-dependent pathway that promotes protein turnover that results in Notch signal inactivation.[52–54]

Vascular remodeling and stabilization

During embryogenesis, formation of the cardiovascular system is an early and essential process. Vascular development initiates with the differentiation of endothelial precursors, the angioblasts, into endothelial cells.[55,56] These cells then gather together to form a primitive vascular network of uniformly sized vessels composed entirely of endothelial cells by the process of vasculogenesis. This rudimentary vascular plexus is then remodeled to form the veins, arteries and capillaries via angiogenesis. Angiogenesis is a multistep process involving the breakdown of the extracellular matrix, sprouting of cells from pre-existing vasculature, survival and proliferation of these cells, migration of cells away from the existing vessels, morphogenesis to form tubes, and recruitment of accessory cells. Disruption of any of the steps of angiogenesis will disrupt the remodeling of the primitive vasculature and often result in similar phenotypes in vivo. The first indications that Notch might function in vascular development came from expression analysis of Notch1[57] and Notch4.[17] In particular, Notch4 was found to be specifically expressed in developing vessels.[16,58]

Studies in mice have demonstrated that Notch function is essential for the remodeling of the rudimentary vascular plexus. It is through these analyses that Notch has been established to be an angiogenic regulator. Mouse embryos deficient for Jagged1, Notch1, Notch1/Notch4, or the presenilins (PS1 and PS2), die between E9.5–10.5 and display severely disorganized vasculature.[37–39] Mice nullizygous for Notch1 die as embryos with severe neuronal and somitic defects.[24,59] However, some Notch1-deficient embryos also display vascular defects resembling, but often less severe than, those observed in the Notch1/Notch4 double nulls.[38] Thus, Notch1 and Notch4 are functionally redundant during vascular development. In Notch or Jagged1 mutant embryos, vasculogenic formation of the head, yolk sac and intersomitic

vessels is unaffected. Instead, there is a failure to reorganize these rudimentary vessels into large vessels and branching capillaries. Transgenic mice in which an activated form of Notch4 is expressed in embryonic endothelium also display similar defects in vascular remodeling and die at E10.5.[60] Therefore, appropriate levels of Notch signaling in endothelial cells are critical for embryonic vascular development. In all these mouse models, initial establishment of the vascular network does not appear to be significantly compromised, indicating that these Notch proteins and ligands are not required for vasculogenesis, but are essential for angiogenesis. Many different signaling pathways (VEGF/ephrin/angiopoietin/PDGF) are essential for the complex process of vascular remodeling to proceed to completion.[61–69] Both in vivo and in vitro data suggest that Notch signaling interfaces with several of these angiogenic signaling pathways.

An important component of vascular remodeling is the endowment of newly formed vessels with accessory cells. These recruited mural cells are essential for vessel stabilization and promote endothelial cell survival. In embryos that express activated Notch4 in endothelial cells, dilation of the aortae is associated with a failure of the recruited vascular smooth muscle cells to organize around the endothelial cells.[60] This phenotype is similar to that observed for mice nullizygous for PDGF-B and PDGFR-β.[63,64] In these PDGF-deficient mice, vasculogenesis does not rely on the recruitment of vascular accessory cells, but the subsequent remodeling does depend on mesenchymal–endothelial cell interactions. Thus, one may speculate that the Notch and PDGF pathways function to direct endothelial–mural cell interactions (Fig. 2A). In contrast to the dilated aortae and anterior cardinal veins of embryos with Notch4 activation, loss of Notch1 or Notch1/Notch4 results in a collapse or discontinuity of these vessels.[38,60] Thus, the failure of Notch mutant embryos to remodel their vasculature may be due partly to defects in the organization of the perivascular cells. Consistent with this hypothesis, Notch3 and Jagged1 are expressed in the vascular smooth muscle cells of the aorta, and expression of Notch1, Notch4 and Jagged2 is restricted to the endothelial cells of E13.5 embryos.[17,70] Notch signaling may provide a mesenchymal–endothelial cell signal that aids in the stabilization of the newly formed vasculature.

The vascular remodeling and aortic defects may either be due to a failure of endothelial cell autonomous Notch signaling or a defect in Notch signaling to neighboring accessory cells. Distinguishing these two potential activities may be difficult as both endothelial and vascular smooth muscle cells express multiple Notch proteins and Notch ligands. Mouse models that allow cell-type-specific mutation of Notch genes in either the endothelial or perivascular cell may help resolve this issue. It is also important to explore the potential collaborative or complementary efforts of the Notch and PDGF signaling pathways in vascular smooth muscle cell organization or stabilization.

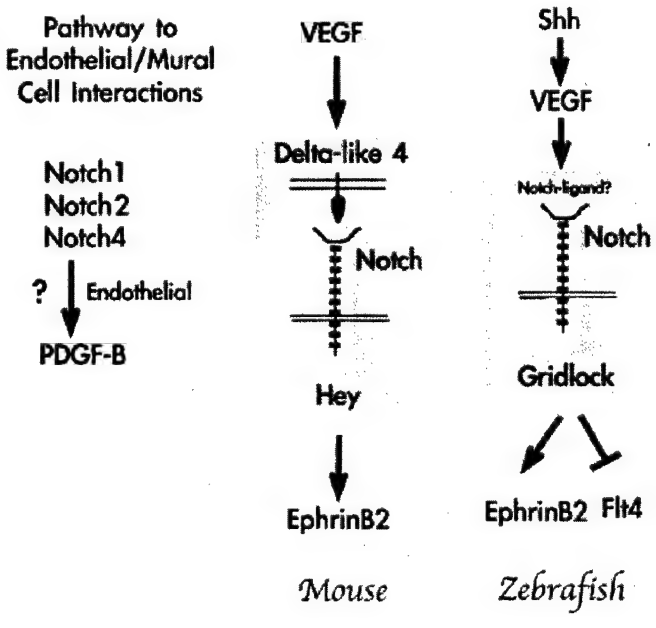

Figure 2. Notch interacts with angiogenic signaling pathways in vasculature. **A:** A speculative model as to how Notch regulates endothelial–mural cell interactions during vascular development based upon the observation that mice mutant for Notch bear similarities to PDGF-B/PDGFR-β mutant mice. Disruption of Notch1 and Notch4, PDGF-B, or PDGFR-β results in a failure of vascular smooth muscle cells to organize around the developing aorta. Similarly, the specialized vascular mural cell of the glomerulus, the mesangial cell, is absent in the kidney of Notch2 or PDGF-B-deficient mice. **B:** Model for the interaction between Notch and angiogenic signaling pathways in arteriogenesis. In human endothelial cells, VEGF induces the expression of Notch and the Notch ligand Delta4, whereas activation of Notch upregulates the expression of ephrinB2. In zebrafish arterial/venous specification, Notch acts genetically downstream of Shh and VEGF; while, Notch activity upregulates ephrinB2 and inhibits flt4 expression.

Arterial/venous specification

Arteries and veins are morphologically, functionally and molecularly unique. The molecular mechanisms governing differentiation and organization of the rudimentary vessels into arteries and veins are just now being uncovered. In mammals, a role for Notch in arterial/venous specification has been inferred from expression studies. Notch family members and Notch ligands are expressed throughout the vasculature early in development, but later become restricted to the arteries. This can be seen most clearly in developing heart outflow tracts and male gonads of the mouse. At embryonic day 9.5, Notch4 expression is first observed in the anterior cardinal vein.[17] By E13.5, Notch4 is absent from the vena cava, which develops from the anterior cardinal vein, but is expressed in the endothelial cells of the aorta.[17,70] In the aorta, Notch4 expression overlaps with Notch2, Jagged1 and Jagged2.[70] In the developing E11.5 male gonad, Notch1, Notch4 and Delta-like4 are expressed in both the venous and arterial endothelial cells.[71] By the following day, the expression of these genes becomes restricted to the arterial vessels. This transition in expression suggests a role for Notch in the regulation of arterial/venous endothelial cell specification or in the maintenance of the arterial phenotype. In fact, recent experiments demonstrate that Notch signaling functions in zebrafish arterial/venous-specification. During development of the zebrafish dorsal aorta, loss of Notch or the downstream Notch-target gene *gridlock* leads to a loss in the arterial cell marker ephrinB2 and an increase in the expression of the venous marker, EphB4.[40,41] Conversely, constitutive activation of Notch suppressed the expression the venous cell marker flt4.[40] Thus Notch, through the induction of gridlock, may promote the development of the embryonic artery and inhibit the differentiation of the vein. Consistent with the zebrafish genetics, ectopic expression of an activated form of Notch4, upregulated ephrinB2 in cultured human microvascular endothelial cells.[72] In fact, the ephrinB2 knockout mice closely resemble mice deficient for Notch1 or Notch1 and Notch4.[61,65] Whether the Notch-specific induction of ephrinB2 also occurs via the mammalian homolog of gridlock, Hey2 still needs to be ascertained. Mice nullizygous for Hey2 do not display defects in arterial specification.[73–75] This may be due to functional redundancy with Hey1, which is often co-expressed with Hey2 in arterial endothelial cells.[50] Consistent with this hypothesis of redundancy, mice nullizygous for both Hey1 and Hey2 display a constriction of the aortae, though not loss of the major arteries.[76]

The use of zebrafish genetics has also led to the identification of genes acting upstream of Notch. These studies demonstrate that Sonic Hedgehog (SHH) is required for the induction of VEGF-A and arterial specification.[77] In turn, VEGF-A induced the expression of Notch, while ectopic expression of Notch rescued ephrinB2 expression in the absence of VEGF-A. Does this cascade also exist in mammals? In a transgenic mouse model, ectopic expression of VEGF-A in the heart preferentially promoted an increase in capillaries that expressed ephrinB2.[78] Moreover, sensory neurons and Schwann cells in vitro, which express VEGF-A endogenously, induce the expression of an arterial marker in isolated embryonic endothelial cells.[79] Thus, similar to zebrafish, VEGF-A may also regulate arterial specification through the upregulation of Notch in mammals. In fact, VEGF-A induced both Notch1 and Delta-like4 in cultured human iliac arterial endothelial cells.[80] Taken together, these data demonstrate that arterial/venous specification occurs via VEGF-A/Notch signaling cascade resulting in the upregulation of the arterial endothelial cell marker ephrinB2 (Fig. 2B). These findings place Notch squarely in the midst of other known angiogenic regulators, in this case VEGF and ephrinB2. Whether these signaling pathways regulate each other directly or indirectly needs to be determined. Our understanding of the molecular mechanisms by which Notch regulates arterial/venous specification may likely provide insights into the pathological angiogenesis that support cancer growth.

Vascular homeostasis

Insights into a function for Notch in vascular homeostasis can be drawn from the human neurovascular disorder, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). In a majority of patients, CADASIL has been found to correlate with missense mutation in Notch3. CADASIL is a late-onset (average age of 45) autosomal dominant disorder characterized by migraines with aura and recurrent strokes that lead to psychiatric symptoms, progressive cognitive decline, dementia, and death.[81] These neuropathological symptoms arise secondary to a slow developing arteriopathy, associated with the disorganization and destruction of the vascular smooth muscle cells surrounding the cerebral arteries and arterioles (Fig. 3). Regression of vascular smooth muscle cells is associated with a decrease in vessel wall thickness, a loss of extracellular matrix and vessel wall weakness.[82] Within the vascular smooth muscle cells, there is an accumulation of the extracellular domain of Notch3 and an abnormal deposition of particles in the extracellular matrix, referred to as granular osmophilic materials (GOM) (Fig. 3).[83] In this disorder, arterial lesions are not restricted to the brain and are found in arteries of the skin and retina.[83–85]

The CADASIL phenotype correlates with the expression of Notch3 in vascular smooth muscle cells.[70,81] The hypothesis being that Notch3 functions to maintain cell–cell interactions or communication between vascular smooth muscle cells and arterial endothelial cells. A recent study has recreated the CADASIL vessel pathology in transgenic mice that express a Notch3 transgene encoding the CADASIL R90C mutation specifically in vascular smooth muscle cells.[86] The vasculature of these mice showed classic CADASIL

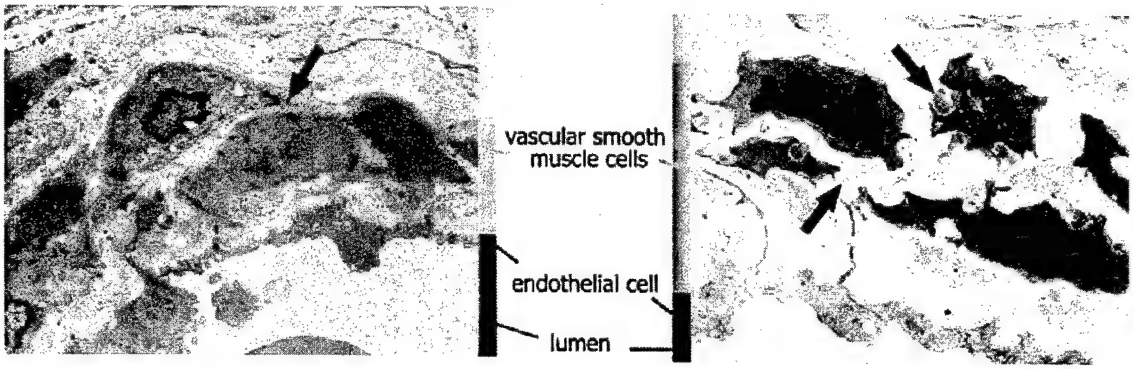

Figure 3. Vessel pathology in CADASIL skin biopsies. Normal vessel in left panel displaying vessel architecture and tight contact between vascular smooth muscle cells (red arrow). CADASIL vessel in right panel displaying poor contact between vascular smooth muscle cells (blue arrow) and GOMs being shed by vascular smooth muscle cells.

arteriopathy, including GOM deposits and Notch3 accumulation. However, these hallmarks were preceded by the disruption of anchorage and adhesion of vascular smooth muscle cells to neighboring cells followed by degeneration of the vascular smooth muscle cells. Thus, CADASIL results from reduced vascular smooth muscle cell contact and viability and the GOM deposition and accumulation of the extracellular domain of Notch3 are secondary consequences of this cellular deterioration. Consistent with a role for Notch3 in cell survival, expression of a constitutively active form of Notch3 in rat aortic smooth muscle cells resulted in the induction of cFlip, an antagonist of Fas-dependent apoptosis.[87] In addition, ectopic expression of Hey1 in cultured vascular smooth muscle cells promoted cell survival via Akt and thus inhibited apoptosis in response to serum starvation and Fas ligand.[88] Taken together, these data indicate that Notch3 maintains arterial vessel homeostasis by promoting vascular smooth muscle cell survival. The resulting arterial vessel wall leakiness could arise from vascular smooth muscle cell death or a failure of vascular smooth muscle cells to communicate to their neighboring endothelial cells. Disruption of Notch3 activity in mice may help define the nature of this defect.

The specific activity of CADASIL mutant Notch3 proteins is still poorly understood. One complication in interpreting mutant Notch3 function arises from conflicting in vitro studies that have shown that truncated cytoplasmic Notch3 can either inhibit or activate the CSL transcription factor.[89,90]

Organ-specific angiogenesis

Studies of the Alagille syndrome (AGS) implicate Notch signaling in organ-specific angiogenesis. In humans, mutations in the Jagged1 gene have been identified in 60–70% cases of AGS, a dominant, pleiotropic developmental disorder with phenotypic abnormalities of the liver, heart, kidney, eye, vertebrae, limbs and facial features. This wide spectrum of defects correlates with the dynamic expression of Jagged1 in the embryo.[91–94] The observed defects in the liver, heart and kidney have notable vascular features. A relationship between Notch signaling and AGS has been further elucidated by the phenotypes of two mouse mutants. Mice homozygous for a hypomorphic allele of Notch2^{del1}, or doubly heterozygous for Notch2^{del1} and Jagged1, phenocopy the hepatic, cardiac and renal defects of AGS (Fig. 4).[93,94] Thus, Notch2 and Jagged1 may function as a ligand/receptor pair during the specialized vascular development in these organs.

Genetic characterization of AGS patients have found that the mutations in Jagged1 include entire gene deletions (6%), protein truncations due to insertions, deletions, nonsense and splice site mutations (82%) and missense mutations (12%).[95] The missense mutations are clustered within the extracellular domain of Jagged1. Patients with large deletions encompassing the entire Jagged1 allele have phenotypes similar to those involving intragenic mutations, indicating that AGS results from Jagged1 haploinsufficiency.[95,96] Consistent with this hypothesis, two Jagged1 AGS missense mutants (R148H and L37S) do not activate Notch using an in vitro assay and instead improperly accumulate in the endoplasmic reticulum.[97] Moreover, mice heterozygous for a null allele of Jagged1 and a hypomorphic allele of Notch2 display many of the same defects as AGS patients.[93,94] In contrast, some patients have been identified with Jagged1 missense mutations that do not have the five major signs of AGS, but present with isolated cardiac or hepatic defects.[98,99] Thus, there may be a correlation between genotype and phenotype for some Jagged1 mutations or some organs require higher levels of Notch activity than others during development.

Most AGS patients are diagnosed with liver disease due to a paucity of bile ducts.[96] A similar liver phenotype has been observed in mice heterozygous for Notch2^{del1}/Jagged1.[94]

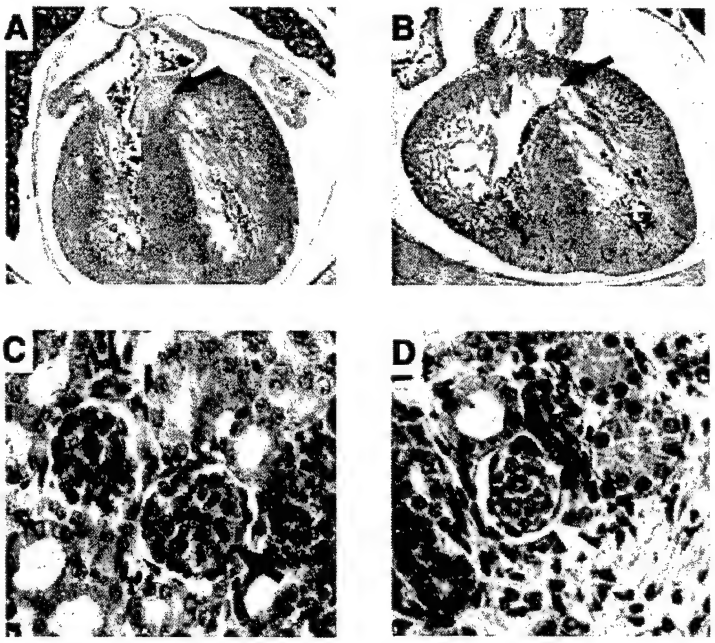

Figure 4. Heart and kidney defects in mice with Jagged1/Notch2 mutations.[93,94] **A:** Heart from wild-type mouse with normal ventricular septum (black arrow). **B:** Ventricular septal defect (black arrow) in heart of Jagged1/Notch2 double heterozygous mice. **C:** Kidney from wild-type mouse with glomerulus (black arrow). **D:** Defective glomeruli in mice homozygous for hypomorphic Notch2 mutation. Glomerulus with capillary aneurism (yellow arrow).

During hepatic development, the hepatoblasts juxtaposed to the portal vein differentiate into epithelial cells of the bile duct.[100] Consistent with Jagged1 functioning in bile duct development, Jagged1 is expressed in the endothelial cells and non-endothelial supporting cells of the portal veins and Notch2 is expressed in the surrounding cells.[94] In the liver, defects in bile duct differentiation may result from the disruption of Jagged1 signaling in endothelial and mural cells of the portal vein to Notch2 in the neighboring epithelial cells that give rise to the bile ducts.

90% of AGS patients present with cardiac malformations including defects in the pulmonic valve, pulmonary artery and its branches, ventricular and atrial septation, aortic stenosis, coarctation, and tetralogy of Fallot.[96,101] These varied defects correlate with the dynamic expression of Notch and Notch ligands in the developing murine heart. At E12.5–13.5, the heart undergoes remodeling of the outflow tract and aortic arch arteries; inter-atrial and inter-ventricular septation is established and early development of the cardiac valves is initiated. At E12.5, Notch1 and Notch2 are expressed in the endothelial cells of the aorta.[91] The following day Notch2 expression is absent in the aorta;[70] while the expression of Notch1 is maintained and overlaps with Notch4, Jagged1 and Jagged2.[70,92] At this embryonic stage, Jagged1 is also co-expressed with Notch2 in the pulmonary artery, walls of the atria and the ventricular myocardium.[70,91,92] Unlike the receptors, the expression of Jagged1 is not restricted to the endothelial cells, but extends into the surrounding vascular mural cells of the aorta and atria.[70,92] Thus, Jagged1 in mural cells may signal to one or more of the Notch proteins expressed in the endothelial cells.

Consistent with its expression in the developing heart, half of Notch2^{del1} homozygous embryos die prior to E16.5 due to severe heart defects.[93] At E11.5, the Notch2^{del1} homozygotes exhibit pericardial effusion and thinning of the myocardial wall surrounding the endocardium. Jagged1 via Notch2 may regulate multiple events during heart development as mice heterozygous for Notch2^{del1} and a Jagged1 null allele display multiple heart-related defects, including right ventricular hypoplasia, narrowing of the pulmonary artery, atrial and ventricular septation defects and dextropositioning of the aorta[94] (Fig. 4). Mice nullizygous for the Notch-target gene, Hey2, display similar heart defects to those observed in Notch2^{del1}/Jagged1 doubly heterozygous mice.[73–75] In support of this relationship, expression of Notch2 coincides with Hey2 in the outflow tracts of the heart.[73–75,93,94] Thus, Jagged1/Notch2 signaling may induce the expression of Hey2 to regulate heart development. Whether the defects in heart development arise from a loss of Notch activity within either the mural or endothelial cells is unclear and should be addressed with tissue-specific disruption of Jagged1 or Notch2.

Renal anomalies also occur in 23–74% of AGS patients studied. Insights into the renal defects in AGS patients may be drawn from mice homozygous for Notch2^{del1} and doubly heterozygous for Notch2^{del1} and Jagged1. Half of the mice

homozygous for Notch2^{del1} survive until birth, but die perinatally from defects in renal glomerular development.[93] Vascularization of the glomeruli begins with migration of endothelial cells into the glomerular cleft. These endothelial cells form capillary loops that branch to form complex capillary tufts. Development of the glomeruli begins in Notch2^{del1} mice, but appears to arrest at the capillary loop stage.[93] In a portion of the glomeruli, the capillary tufts are absent; while, in other glomeruli, the capillary tufts display what seem to be capillary aneurysms (Fig. 4). In these mutant glomeruli, specialized vascular smooth muscle cells, mesangial cells, are entirely absent. One can speculate that this loss leads to a decrease in integrity or stabilization of the vascular beds. These capillary "aneurysm-like" structures have also been observed in mice nullizygous for PDGF-B and PDGFR-β.[63,64] In the PDGF-B and PDGFR-β mice, these structures arise due to a disruption in mesangial cell differentiation. In fact, PDGFR-β-positive mural cells are not recruited into the developing glomeruli of Notch2^{del1} homozygous mice.[93] As for the aorta, a relationship may exist between the Notch and PDGF pathways that facilitates proper endothelial–mural interaction in the kidney (Fig. 2A). Mice heterozygous for Notch2^{del1} and Jagged1 have a similar but less severe renal phenotype, suggesting that Jagged1 functions as a ligand for Notch2 in the vascularization of the renal glomeruli.[94] In fact, Jagged1 is expressed in endothelial cells and/or mesangial cells and Notch2 is expressed in surrounding podocyte precursors.[93]

The defects in the liver, heart and kidneys of AGS patients strongly indicate that Jagged1 functions in organ-specific angiogenesis. However, the pleiotropic phenotype of AGS also demonstrates a requirement for Jagged1/Notch signaling in other cell types besides endothelial and mural cells, such as those that form the vertebrae, eyes, limbs and face.

Concluding comments

In addition to roles for Notch in the embryo and adult arterial vessels, Notch signaling may function during other types of physiological and pathological angiogenesis in the adult. In mice nullizygous for presenilin 2, a protease that functions in Notch activation, hemorrhaging and apoptotic cell death of epithelial and vascular endothelial cells is observed within the lung.[37] In a murine wound model, an increase in Jagged1 expression is observed in the proliferating endothelial cells.[102] Similarly, Delta-like4 expression is upregulated in the invading vasculature found in human tumor xenografts in mice and in the blood vessels of human kidney and breast cancers.[103] Thus, Notch signaling is potentially implicated in angiogenesis in both physiological and pathological angiogenesis. Determination of roles for Notch may benefit from insights

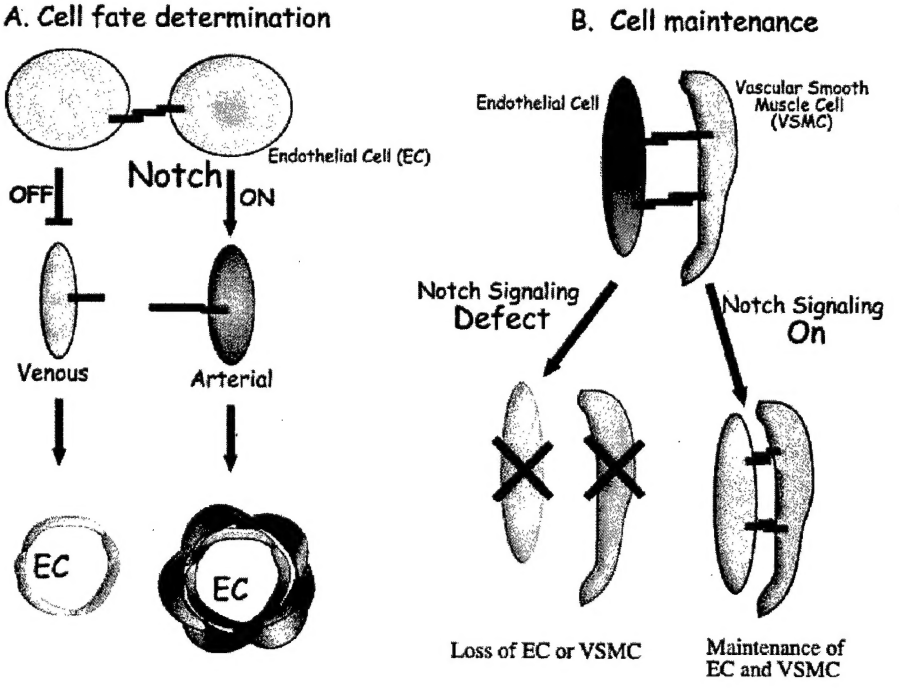

Figure 5. Two models for Notch function in the vasculature. **A:** Cell-fate determination: as seen during arterial/venous differentiation. **B:** Cell maintenance: communication between vascular cells required to maintain cell viability.

into the mechanism by which Notch functions in angiogenesis, vessel specification and vessel homeostasis.

It is attractive to pair the observation that Notch regulates binary cell-fate decisions with the recent experimental implication of Notch function in arterial/venous endothelial cell specification (Fig. 5). In zebrafish, arterial/venous specification occurs via a SHH–VEGF-A–Notch–ephrinB2 regulatory cascade. Studies in mammals are beginning to confirm that portions of this pathway may also function during arterial/venous specification. Cell lineage analysis in *Xenopus* and zebrafish suggests that specification of arterial and venous endothelial cells may occur as early as the angioblast stage prior to the formation of the primitive vascular plexus.[41,104] If this is the case, does Notch function in the early endothelial cell precursor to establish the arterial and venous cell fates? This implies that arterial/venous specification is not essential for vasculogenesis, but may be required for angiogenesis to progress. If this is the case, then defects in vascular remodeling in mutant Notch embryos may arise because of a failure of early arterial/venous endothelial cell specification.

A common theme that emerges from studies of Notch defects in mice and humans is the absence or loss of cells within the vasculature, such as the mesangial cells in Notch2^{del1} homozygous mice, and the vascular smooth muscle cells in CADASIL. This points to a role for Notch in maintenance or stabilization of the cells that contribute to blood vessels, possibly at stages after key cell-fate decisions have been made (Fig. 5). During angiogenesis, these accessory cells are not only essential for the stabilization of the vasculature, but are also necessary for vascular remodeling. Does this reflect subtleties in cell-fate decisions gone awry, or unexpected functions for Notch in organizing and maintenance of accessory cells in the vasculature? These functions dovetail well with the known functions of PDGF-B and PDGFR-β; however, the relationship between Notch signaling and these signaling cascades has yet to be defined.

Both cell-fate determination and maintenance of endothelial–mural interactions must depend on cell-contact-dependent signaling, a form of signaling in which Notch proteins specialize. Future progress in understanding Notch function in the vasculature may depend on knowing more about the timing and mechanism of vessel specification. Looking to new frontiers in Notch research, one anticipates discoveries relating to the genesis of endothelial and smooth muscle cells, the differentiation and mobilization of adult endothelial stem cells, and the re-awakening of blood vessel growth in tumors.

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